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REGULAR PAPER

Phylogeny and biogeography of the genus *Stevia* (Asteraceae: Eupatorieae): an example of diversification in the Asteraceae in the new world

Akiko Soejima[1] · Akifumi S. Tanabe[2] · Izumi Takayama[3] · Takayuki Kawahara[4] · Kuniaki Watanabe[2] · Miyuki Nakazawa[3] · Misako Mishima[5] · Tetsukazu Yahara[3]

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Abstract The genus *Stevia* comprises approximately 200 species, which are distributed in North and South America, and are representative of the species diversity of the Asteraceae in the New World. We reconstructed the phylogenetic relationships using sequences of ITS and cpDNA and estimated the divergence times of the major clade of this genus. Our results suggested that *Stevia* originated in Mexico 7.0–7.3 million years ago (Mya). Two large clades, one with shrub species and another with herb species, were separated at about 6.6 Mya. The phylogenetic reconstruction suggested that an ancestor of *Stevia* was a small shrub in temperate pine–oak forests and the evolutionary change from a shrub state to a herb state occurred only once. A Brazilian clade was nested in a Mexican herb clade, and its origin was estimated to be 5.2 Mya, suggesting that the migration from North America to South America occurred after the formation of the Isthmus of Panama. The species diversity in Mexico appears to reflect the habitat diversity within the temperate pine–oak forest zone. The presence of many conspecific diploid–polyploid clades in the phylogenetic tree reflects the high frequency of polyploidization among the perennial *Stevia* species.

Keywords Agamospermy · Asteraceae · Biogeography · Divergence time · Phylogeny · Polyploidy

Introduction

Asteraceae is the most species-rich family in flowering plants, and thus the mechanisms behind its diversification have been of continued interests in evolutionary biology (Bremer 1994; Funk et al. 2005; Heywood 2009). Previous studies revealed that adaptive divergence, polyploidization, hybridization and geohistorical factors contributed to the diversification of Asteraceae as in other groups of flowering plants (Baldwin and Sanderson 1998; Gottlieb 1981, 2004; Rieseberg and Willis 2007; Rieseberg et al. 2003; Soltis et al. 2004). Among them, some recent molecular phylogenetic studies suggested that geohistorical factors might have driven rapid speciation in many plant groups during the late Tertiary, i.e., since approximately 10 million years ago (Liu et al. 2006; Richardson et al. 2001; Willis and Whittaker 2002). To test this suggestion, further molecular phylogeographic studies in species rich areas are needed (Liu et al. 2006).

The purpose of this study is to provide a molecular phylogenetic study on the genus *Stevia* in Mexico, an area rich in species of Asteraceae. In the family Asteraceae distributed across the world, its species richness is the highest in the New World including Mexico (Bremer 1994; Crawford et al. 2014; Funk et al. 2005, 2009a; Panero and Crozier

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✉ Akiko Soejima
soejima@sci.kumamoto-u.ac.jp

[1] Department of Biological Science, Faculty of Advanced Science and Technology, Kumamoto University, Kumamoto 860-8555, Japan

[2] Department of Biology, Graduate School of Science, Kobe University, Kobe 657-8501, Japan

[3] Department of Biology, Graduate School of Science, Kyushu University, Fukuoka 819-0395, Japan

[4] Hokkaido Research Center, Forestry and Forest Products Research Institute, Sapporo 062-8516, Japan

[5] University Museum, Kyushu University, Fukuoka 812-8581, Japan

2016). According to the Asteraceae metatree constructed by Funk et al. (2009b), the Heliantheae Alliance, a large group of ca. 5700 spp. including *Stevia* (Eupatorieae), forms a core of diversity in the New World. The origin of the Heliantheae Alliance was supposed to be 25–35 Mya based on pollen data (Funk et al. 2009b; Graham 1996). The area optimization analysis based on the Asteraceae metatree (Funk et al. 2009b) suggested that the Heliantheae Alliance originated and diverged in North America including Mexico, and repeatedly migrated into South America where further diversifications took place. However, only a few studies have been made on the diversification process of this group (Rivera et al. 2016). Among them, for *Brickellia* of Eupatorieae, Schilling et al. (2015) estimated its origin as 9 Mya and showed that it actively diversified from late Miocene to present in Mexico under the climatic and geological changes. To elucidate the mechanisms behind the diversification of the Heliantheae Alliance, further phylogenetic studies on other groups are needed.

Here, we estimated divergence times from a phylogeny of the genus *Stevia*, one of the most distinctive genera in Eupatorieae, which is characterized by heads with five florets and a dense pubescence on the inner surface of the corolla throat, as well as a distinctive anther appendage (Grashoff 1972; King and Robinson 1987; Robinson et al. 2009). *Stevia* is a genus endemic to the New World, including 175–230 species distributed widely from southern United States in North America to northern Patagonia of Argentina in South America (Gutiérrez et al. 2016; Hind and Robinson 2007), but is highly diversified in Mexico where approximately 100 species have been identified (Soejima et al. 2001b; Turner 1997). Mexican species of *Stevia* exhibit notably high diversity in plant habit (annual, perennial, and shrub) (Fig. 1), chromosome number ($n=4$, 10, 11, and 12; diploid, and polyploid), and reproductive mode (sexual reproduction, vegetative reproduction, and agamospermy) (Grashoff 1972; King and Robinson 1987; Watanabe et al. 2001), providing an extraordinary opportunity for studying the mechanisms behind species diversification. By contrast, although approximately 125 species of *Stevia* have been described from South America (King and Robinson 1987), those show much lower diversity in life form (perennials only), chromosome number ($x=11$ only; Watanabe et al. 2007), and reproductive mode (mostly sexual; Grashoff 1972). Thus, we focus on the diversity of *Stevia* in Mexico but also included some species of Brazil to determine where *Stevia* originated. By reconstructing phylogeny of the species of *Stevia* in Mexico and Brazil and determining divergence time of major clades, we will address the following questions. (1) Where did the genus *Stevia* originate and when did it migrate between American continents? (2) Which species is placed in the most ancestral branch? (3) What are the most ancestral states of life form and chromosome number and how often did life form and chromosome number change? (4) How often did agamospermous polyploids evolve from sexual diploids? (5) What are implications of new findings on our understanding of the evolutionary history of Asteraceae in general?

Materials and methods

Taxon sampling

All of the samples used in this study are shown in Table 1. We collected 70 species of *Stevia* in Mexico, which represent approximately 65% of the known Mexican species. Voucher specimens are kept in MEXU, while some duplicate specimens are distributed to KYO, TEX, and MO. In addition, 13 species were collected in Brazil as representatives of the South American species, which covered about 10% of the species known in South America. Voucher specimens of the Brazilian samples are kept in the Goro Hashimoto Herbarium (São Paulo, Brazil), while some duplicates are housed at MO.

Among the Mexican *Stevia*, 41 perennial species are known to include agamospermous polyploids (Grashof 1972; Watanabe et al. 2001). For these species, we made efforts for finding sexually reproducing diploid populations to elucidate divergence at the diploid level (Soejima et al. 2001a; Watanabe et al. 2001). We found both sexual diploid and agamospermous polyploid populations for 19 species and determined the DNA sequences of both sexual and agamospermous plants for 9 of them. We used *Carphochaete grahamii* A. Gray and *Revealia macrocephala* (Paray) R. M. King and H. Rob. as outgroups due to their close similarity to *Stevia* (King and Robinson 1987). *Carphochaete* is a small genus comprising only five species. *Revealia* is monotypic (King and Robinson 1987), or has been merged with *Carphochaete* (Turner 1997). These two genera are endemic to Mexico. For additional outgroups, we obtained the sequence data of the following five species from GenBank: *Ageratina wrightii* (JQ737035), *Hofmeisteria schaffneri* (AF374907), *H. urenifolia* (AF374906), *Mikania micrantha* (DQ826453), and *M. scandens* (AF177783 and AF177823). They were selected from closely related genera based on the Asteraceae meta-tree (Funk et al. 2009b).

DNA extraction, amplification, and sequencing

DNA from all samples was extracted from silica gel-dried leaves using the CTAB method (Doyle and Doyle 1987). A nuclear ribosomal region, which included two internal transcribed spacers, was amplified using two pairs of primers: ITS2 and ITS5, and ITS3 and ITS4 (White et al. 1990). The PCR cycles comprised an initial denaturation at 95 °C for

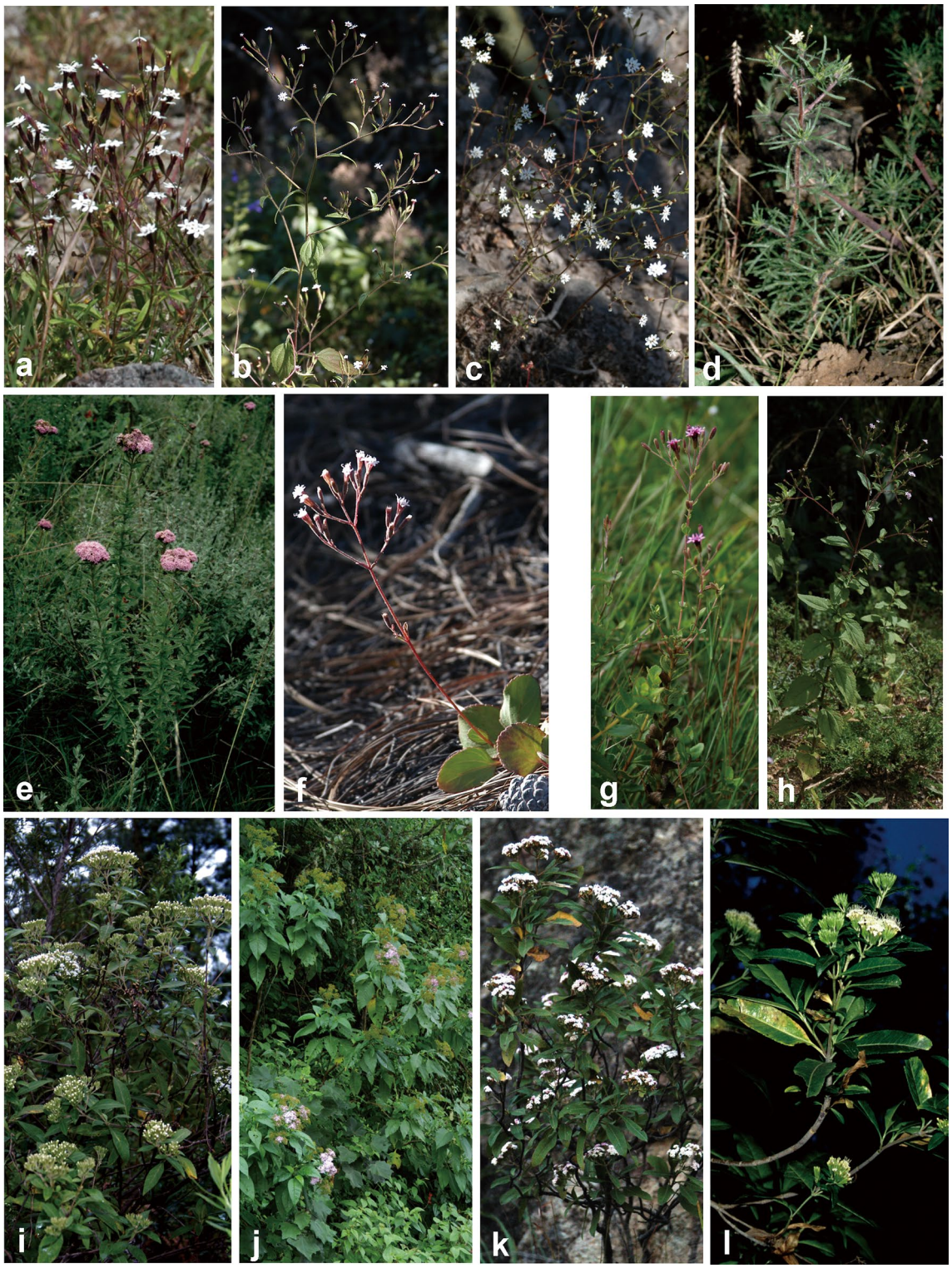

Fig. 1 Diversity of *Stevia* species. **a** *S. ephemera* (annual, $n=4$, Mexico), **b** *S. aschenborniana* (annual, $n=12$, Mexico), **c** *S. occidentalis* (annual, $n=10$, Mexico), **d** *S. mexicana* (annual, $n=11$, Mexico), **e** *S. eupatoria* (perennial, $n=12$, Mexico), **f** *S. liebmanii* (perennial, $n=11$, Mexico), **g** *S. veronicae* (perennial, $n=11$, Brazil), **h** *S. clausseni* (perennial, $n=11$, Brazil), **i** *S. subpubescens* (shrub, $n=12$, Mexico), **j** *S. microchaeta* (shrub, $n=12$, Mexico), **k** *S. seleriana* (shrub, $n=12$, Mexico), **l** *S. nelsonii* (shrub, $n=12$, Mexico)

Table 1 Samples from Mexico and Brazil

Taxon	*Voucher*	Locality	Chromosome number	ITS	*matK*	*psbC-trnS*	*rpl16–rpl14*	*rps14-psaB*	*rps18–rpl20*
Samples from Mexico									
S. alatipes B. L. Rob.	Y0421	Jalisco: 19.5 km N of Mascota	$2n=33$	AB457235	–	–	–	–	–
	Y0488	Jalisco: 4 km SE of JCT from Jilotlan via San Ishidro	$2n=22$	AB457234	AB457331	AB457383	AB457430	AB457477	AB457527
S. anadenotricha (B. L. Rob) Grashoff	Y0434	Jalisco: 30 km N of Mascota		AB457237	AB457333	AB457385	AB457432	AB457479	AB457529
S. aschenborniana Sch. Bip. ex Klotzsch	Y0243	Guerrero: 11.7 km W of JCT from Taxco to Ixcateopan	$2n=24$	AB457238	AB457334	AB457386	AB457433	AB457480	AB457530
S. berlandieri A. Gray	Y0712	Nuevo Leon: 36 km S of Monterrey		AB457239	AB457335	AB457387	AB457434	AB457481	AB457531
S. caracasana DC.	Y01218	Jalisco: 3.8 km SE of JCT to Route 110	$2n=22+1\sim3B$	AB457240	–	–	–	–	–
	Y0249	Guerrero: 15.9 km E of Tixtla de Guerrero	$2n=33$	AB457241	–	–	–	–	–
S. chiapensis Grashoff	Y0626	Chiapas: 22 km NW of Motozintla de Mendoza		AB457242	–	–	–	–	–
S. connata Lag.	Y0043	Guerrero: 29 km W to Taxco	$2n=33$	AB457245	–	–	–	–	–
	Y0257	Guerrero: 13.8 km E of Chilapa	$2n=22$	AB457244	AB457339	AB457391	AB457438	AB457485	AB457535
S. decumbens (B. L. Rob. and Greenman) E. Greene	Y0318	Oaxaca: 134 km S from Puebla–Oaxaca border on Hwy135 Cuota		AB457246	–	–	–	–	–
S. deltoidea E. Greene	Y0273	Guerrero: 49 km SW from JCT of the Hwy 95 Libre to Filo de Caballo	$2n=33+0\sim3B$	–	–	–	–	–	–
	Y0332	Oaxaca: 17.8 km NE from JCT on Route 175 to Valle National	$2n=33+0\sim3B$	AB457247	AB457340	–	–	–	–
S. dictyophylla B. L. Rob.	Y0801	Jalisco: 1.6 km W of El Pueblito	$2n=24$	AB457248	AB457341	AB457392	AB457439	AB457486	AB457536
S. ecatepecana Soejima, Yahara and Watanabe	Y1111	Oaxaca: 23.5–24.2 km S to Santa Maria Ecatepec from JCT Mex 190		–	–	–	–	–	–
	Y1038	Puebla: 14 km NE of Teotitlan	$2n=12II$	AB457249	AB457342	AB457393	AB457440	AB457487	AB457537
S. elatior Kunth	YI102	Oaxaca: 43.2 km SE from Nochixtlan		AB457250	–	–	–	–	–
S. ephemera Grashoff	Y0313	Oaxaca: 75 km S from Puebla–Oaxaca border on Hwy 135 Cuota	$2n=8$	–	–	–	–	–	–
	Y1043	Oaxaca: 5.7 km SW of Suchixtlahuaca	$2n=4II$	AB457251	AB457343	AB457394	AB457441	AB457488	AB457538

Table 1 (continued)

Taxon	*Voucher*	Locality	Chromosome number	ITS	*matK*	*psbC-trnS*	*rpl16–rpl14*	*rps14-psaB*	*rps18–rpl20*
S. eupatoria (Sprengel) Willd.	Y0006	Hidalgo: 11 km from Pachuca to El Chico	$2n=24$	AB457252	–	–	–	–	–
	YO451	Hidalgo: near the entrance of National Park	$2n=48$	AB457253	–	–	–	–	–
S. filodeca-balloana Soejima, Yahara and Watanabe	Y0287	Guerrero: 8.5 km SW of Filo de Caballo	$2n=22$	AB457254	AB457344	AB457395	AB457442	AB457489	AB457539
S. glandulosa Hook. and Arn.	Y0464	Jalisco: 27 km W of Talpa de Allende		AB457255	–	–	–	–	–
S. hypomalaca B. L. Rob.	Y0101	Mexico: 6 km NE of Temas-caltepec		AB457256	AB457345	AB457396	AB457443	AB457490	AB457540
S. iltisiana Grashoff	Y0114	Mexico: 9 km W of Rio Frio		AB457257	–	–	–	–	–
S. incognita Grashoff	Y0304	Oaxaca: 40 km S from Puebla Oaxaca border on Hwy 135 Cuota		AB457258	–	–	–	–	–
S. isomeca Grashoff	Y0113	Mexico: 9 km W of Rio Frio	$2n=33+1\sim3B$	AB457259	–	–	–	–	–
S. jaliscensis B. L. Rob.	Y0387	Jalisco: 11 km N of Guadalajara		AB457260	–	–	–	–	–
S. jorullensis Kunth	Y0308	Oaxaca: 75 km S from Puebla–Oaxaca border on Hwy 135 Cuota	$2n=24$	AB457261	–	–	–	–	–
S. karwinskyana Steudel	Y0523	Veracruz: Dos Hermonas, 6 km NW of Acajete on Route 140	$2n=33$	AB457265	–	–	–	–	–
	Y0555	Veracruz: Tenampa	$2n=22$	AB457264	–	–	–	–	–
S. latifolia Benth	Y0236	Guerrero: 10 km N of JCT to Ixcateopan	$2n=33$	–	–	–	–	–	–
	Y1015	Mexico: 13.5 km N of Temas-caltepec	$2n=33$	AB457266	AB457346	AB457397	AB457444	AB457491	AB457541
S. lehmannii Hieron.	Y0299	Guerrero; 30.8 km S of Petaquillas	$2n=22$	AB457267	AB457347	AB457398	AB457445	AB457492	AB457542
S. lemmonii A. Gray	Y1267	Sinaloa: 92.3 km NE on Mex 40 from Vila Union	$2n=24$	AB457268	AB457348	AB457399	AB457446	AB457493	AB457543
S. liebmannii Sch. Bip. ex Klatt	Y0324	Oaxaca: 13.5 km NE from JCT on Route 175 to Valle National	$2n=33$	AB457270	–	–	–	–	–
S. lita Grashoff	Y0250	Guerrero: 19.5 km E of Tixtla de Guerrero	$2n=20$	AB457271	AB457350	AB457401	AB457448	AB457495	AB457545
S. lucida Lag. var. *oaxacana* (DC.) Grashoff	YI171	Oaxaca: 3.8 km N from Guelatao, on Hwy 175		AB457272	–	–	–	–	–
S. mascotensis Soejima and Yahara	Y0420	Jalisco: 19.5 km N of Mascota	$2n=44$	-	AB457351	–	–	AB457496	AB457546

Table 1 (continued)

Taxon	*Voucher*	Locality	Chromosome number	ITS	*matK*	*psbC-trnS*	*rpl16–rpl14*	*rps14-psaB*	*rps18–rpl20*
S. mexicana Soejima, Yahara and Watanabe	Y1011	Mexico: 21.5 km NE of Tejupilco	$2n=11II$, $2n=22$	AB457274	AB457352	AB457402	AB457449	AB457497	AB457547
S. micradenia B. L. Rob.	Y0270	Guerrero: 42 km SW from JCT of Hwy 95 Libre to Filo de Caballo		AB457275	–	–	–	–	–
S. micrantha Lag.	YO404	Mexico: Reserve adjacent to the Centro de Ecologia in UNAM		AB457276	AB457353	AB457403	AB457450	AB457498	AB457548
S. microchaeta Sch. Bip.	YI251	Oaxaca: 54.3 km N from Guelatao, on Hwy 175	$2n=24$	AB457277	AB457354	–	–	–	–
S. mitopoda B. L. Rob.	Y1022	Puebla: 21.4 km SE of Izcar de Matamoros	2*n*-24	AB457278	AB457355	AB457404	AB457451	AB457499	AB457549
S. monardifolia Kunth	Y0435	Jalisco: 30 km N of Mascota	$2n=22$	AB457280	–	–	–	–	–
	Y0502	Jalisco: 20 km W of JCT with Route 110 to Nevado de Colima	$2n=22$	AB457281	–	–	–	–	–
	Y0823	Veracruz: Perote	$2n=33$	AB457284	–	–	–	–	–
	Y0842	Morelos: 4 km N of Tres Marias	$2n=33+0\sim1B$	AB457285	–	–	–	–	–
	Y0991	Mexico: 2 km NE on Mex 134 from JCT to Sultepec	$2n=33$	AB457286	–	–	–	–	–
	Y0994	Mexico: 2 km NE on Mex 134 from JCT to Sultepec	$2n=33$	AB457283	–	–	–	–	–
	Y1002	Mexico: 6-6.5 km NE on Mex 134 from San Francisco de los Ranchos	$2n=22$	AB457279	AB457356	AB457405	AB457452	AB457500	AB457550
	Y1017	Mexico: 2.4 km W of exit to La Marquera, on Hwy15	$2n=33$	AB457287	–	–	–	–	–
	Y1096	Oaxaca: 16.7 km N from Ixtran to Valle National on Mex 175	$2n=22$	AB457282	–	–	–	–	–
S. myricoides McVaugh	Y0445	Jalisco: 3 km E from JCT of Talpa de Allende-LaCuesta route		AB457288	–	–	–	–	–
S. nelsonii B. L. Rob.	Y0501	Jalisco: 20 km W of JCT with Route 110 to Nevado de Colima	$2n=24$	–	AB457358	AB457407	AB457454	AB457502	AB457552
S. nepetifolia Kunth	Y0857	Guerrero: 42 km SW from JCT with Hwy 95, between Chilpancingo and Filo de Caballo	$2n=36$	AB457289	–	–	–	–	–

Table 1 (continued)

Taxon	*Voucher*	Locality	Chromosome number	ITS	*matK*	*psbC-trnS*	*rpl16–rpl14*	*rps14-psaB*	*rps18–rpl20*
	Y0983	Michoacan: 10 km W of Ucareo	$2n=48$	AB457290	–	–	–	–	–
S. occidentalis (Grashoff) Soejima, Yahara and Watanabe	Y0917	Sinaloa: 25 km S of El Palmito on Mex 40	$2n=20$	AB457291	AB457359	AB457408	AB457455	AB457503	AB457553
S. origanoides Kunth	Y0244	Guerrero: 15.9 km E of Tixtra de Guerrero on Route 93	$2n=22$	AB457293	AB457362	–	–	–	–
	Y0929	Zacatecas: between Jalpa and Tlaltenango	$2n=33$	AB457294	–	–	–	AB457506	AB457556
S. ovata Willd.	Y0290	Guerrero: 39 km SW from JCT of Hwy 95 Libre to Filo de Caballo	$2n=33$	AB457295	–	–	–	–	–
S. perfoliata Cronq.	Y0063	Guerrero: El Fresno Micro-ondes station	$2n=22$	AB457296	AB457363	AB457411	AB457458	AB457507	AB457557
S. pilosa Lag.	YO413	Mexico: 80 km from Mexico to Tulancingo		AB457298	–	–	–	–	–
	Y0841	Morelos: 4 km N of Tres Marias		AB457297	–	–	–	–	–
S. plummerae A. Gray	Y0743	Chihuahua: 17.8 km E of Guadalupe y Calvo	$2n=22+1\sim2B$	AB457299	–	–	–	–	–
	Y0788	Durango: 19.8 km W from El Salto on Mex 40	$2n=44$	-	-	-	-	-	-
S. polycephala Bertol.	Y0140	Oaxaca: 16 km N of Diaz Ordes		AB457300	–	–	–	–	–
S. reticulata Grashoff	Y0412	Jalisco: 49 km W of Ameca, on Route 90		AB457303	–	–	–	–	–
	Y1246	Nayarit: NW slope of Volcan Sanganguey	$2n=22$	AB457302	AB457365	AB457413	AB457460	AB457509	AB457559
S. revoluta B. L. Rob.	Y1026	Puebla: 29.1 km S of Acatepec	$2n=24$	AB457304	AB457366	AB457414	AB457461	AB457510	AB457560
S. rosei B. L. Rob.	Y0905	Durango: 5.8 km W of La Ciudad on Mex 40	$2n=44$	AB457305	–	–	–	–	–
S. rzedowskii McVaugh	Y0649	Zacatecas: 22.4 km W of JCT to Tlaltenango on Mex 70		AB457306	–	–	–	–	–
S. salicifolia Cav.	Y0543	Veracruz: 11 km S from JCT with Route 140 in Guadalupe Victoria		AB457307	–	–	–	–	–
S. scabrella Benth.	Y0892	Durango: 42.1 km SW of San Juan de Michis	$2n=24$	AB457308	AB457367	AB457415	AB457462	AB457511	AB457561
S. scabrelloides Soejima and Yahara	Y0748	Sinaloa–Durango border: 91.8 km SW on Mex 24 from JCT to Guadalupe y Calvo		AB457309	–	–	–	–	–

Table 1 (continued)

Taxon	*Voucher*	Locality	Chromosome number	ITS	*matK*	*psbC-trnS*	*rpl16–rpl14*	*rps14-psaB*	*rps18–rpl20*
S. seemanioides Grashoff	Y0237	Guerrero: 14.8 km N of JCT to Ixtateopan, from Taxco to Tetipex	$2n=22$	AB457311	AB457369	AB457417	AB457464	AB457513	AB457563
S. seemannii Sch. Bip.	Y0325	Oaxaca: 13.5 km NE from Oaxaca to Valle National		AB457310	AB457368	AB457416	AB457463	AB457512	AB457562
S. seleriana B. L. Rob.	Y0375	Oaxaca: 6 km SE of El Cameron		AB457312	AB457370	AB457418	AB457465	AB457514	AB457564
S. serrata Cav.	Y0877	San Luis Potosi: 24.5 km SW of San Luis Potosi	$2n=33$		–	–	–	–	–
	Y0880	Zacatecas: 76 km SW of San Luis Potosi	$2n=33$	AB457313	AB457372	AB457420	AB457467	AB457516	AB457566
S. stolonifera Yahara and Soejima	Y0494	Jalisco: 20 km SE of JCT with Route 110 to Jilotlan via San Ishidro	$2n=22$[a]	AB457315	–	–	–	–	–
	Y0963	Michoacan: 33.4 km NW of Uruapan	$2n=33$[a]	AB457314	-	-	-	-	-
S. subpubescens Lag.	Y0203	Morelos: Mt. Zempoala		AB457316	AB457373	AB457421	AB457468	AB457517	AB457567
S. tephra B. L. Rob.	Y0656	San Luis Potosi: 4 km N of JCT to Guadalcazar and to El Realjo	$2n=33+0\sim3B$	AB457317	–	–	–	–	–
S. tephrophylla S. F. Blake	Y0377	Oaxaca: 1–2 km N of Santo Domingo Petapa		AB457318	AB457375	AB457423	AB457470	AB457519	AB457569
S. tomentosa Kunth	Y0875	Mexico: 65 km NW from Mexico City on Hwy 57	$2n=33+0\sim3B$	AB457319	–	–	–	–	–
S. trifida Lag.	Y0062	Guerrero: 24 km S of Chilpancingo on Hwy 95		AB457320	AB457376	AB457424	AB457471	AB457520	AB457570
S. triflora DC.	Y0971	Michoacan: 10 km N of Acahuato	$2n=36$	AB457321	–	–	–	–	–
S. velutinella Grashoff	Y0055	Guerrero: 13 km SW of Filo de Caballo to Puerto del Gallo	$2n=24$	AB457322	AB457377	AB457425	AB457472	AB457521	AB457571
S. venosa A. Gray	Y0737	Chihuahua–Durango border: on Mex 24		AB457323	–	–	–	–	–
S. vernicosa Greenman	Y1308	Morelos: San Juan Tlacotenco		AB457324	–	–	–	–	–
S. viscida Kunth	Y0407	Jalisco: 23 km W of Ameca		AB457326	AB457379	–	–	AB457523	AB457573
S. zacatecana McVaugh	Y0653	Zacatecas: 28.8 km W of JCT of Mex 54 and Mex 70		AB457327	–	–	–	–	–
S. zephyrantha Grashoff	YS205	Guerrero: near Filo de Caballo		AB457328	–	–	–	–	–
Samples from Brazil									
S. alternifolia Hieron.	Br190	Paraná: 3 km W of Palmeira	$2n=11II+0\sim1B$, $2n=22$	AB457236	AB457332	AB457384	AB457431	AB457478	AB457528
S. cinerascens Sch. Bip. ex Baker	Br251	Santa Catarina: Rio Caveiras	$2n=11II$, $2n=22$	–	AB457336	AB457388	AB457435	AB457482	AB457532

Table 1 (continued)

Taxon	*Voucher*	Locality	Chromosome number	ITS	*matK*	*psbC-trnS*	*rpl16–rpl14*	*rps14-psaB*	*rps18–rpl20*
S. clausseni Sch. Bip. ex Baker	Br318	Santa Catarina: 4 km E of Bom Jardim da Serra		AB457243	AB457337	AB457389	AB457436	AB457483	AB457533
S. commixta B. L. Rob.	Br133	São Paulo: Campos do Jordão		–	AB457338	AB457390	AB457437	AB457484	AB457534
S. leptophylla Sch. Bip. ex Baker	Br151	Paraná: W of Curitiba, near junction of BR 277 and 376 in São Luis Purunã	$2n=22+0\sim1B$	–	–	–	–	–	–
	Br223	Paraná: E limit of Lapa	$2n=11II$	AB457269	AB457349	AB457400	AB457447	AB457494	AB457544
S. lundiana DC.	Br130	São Paulo: Campos do Jordão		AB457273	–	–	–	–	–
S. myriadenia Sch. Bip. ex Baker	Br308	Santa Catarina: Cascata do Avencal	$2n=11II$, $2n=22$	-	AB457357	AB457406	AB457453	AB457501	AB457551
S. ophryophylla B. L. Rob.	Br220	Paraná: E limit of Lapa	$2n=11II$, $2n=22$	-	AB457360	AB457409	AB457456	AB457504	AB457554
S. organensis Gardner	Br138	São Paulo: Pedra do Baú		AB457292	AB457361	AB457410	AB457457	AB457505	AB457555
S. rebaudiana (Bertoni) Bertoni		cultivated in Japan		AB457301	AB457364	AB457412	AB457459	AB457508	AB457558
S. selloi (Sprengel) B. L. Rob.	Br191	Paraná: 3 km W of Palmeira	$2n=11II$	–	AB457371	AB457419	AB457466	AB457515	AB457565
S. tenuis Hook. and Arn.	Br321	Santa Catarina: between Bom Jardim da Serra and Lauro Müller		–	AB457374	AB457422	AB457469	AB457518	AB457568
S. veronicae DC.	Br170	Paraná: Near E limit of Guarapuava	$2n=11II$	AB457325	AB457378	AB457426	AB457473	AB457522	AB457572
	Br204, 205	Paraná: 3 km W of São Luis do Purunã	$2n=11II$, $2n=22$	–	–	–	–	–	–
	Br250	Santa Catarina: Rio Caveiras	$2n=11II$	–	–	–	–	-	–
Outgroups									
Carphochaete grahamii A. Gray	Y0469	Jalisco: 16 km SE of Los Volcanes, on the way to Ayutla		AB457329	AB457380	AB457427	AB457474	AB457524	AB457574
Cronquistia pringlei (S. Watson) R. M. King	Y0735	Chihuahua–Durango border: on Mex 24	$2n=24$	–	AB457381	AB457428	AB457475	AB457525	AB457575
Revealia macrocephala (Paray) R. M. King and H. Rob.	Y0056	Guerrero: 76 km SW of Fillo de Caballo	$2n=22$	AB457330	AB457382	AB457429	AB457476	AB457526	AB457576

Taxa and accessions used for analysis and acession numbers in DDBJ

Chromosome numbers are reported in Watanabe et al. 2001, 2007 and Soejima et al. 2001a

[a]Present count, not published

1 min, followed by 30 cycles for 1 min at 94 °C, 1 min at 50 °C, and 2 min at 72 °C, with a final extension for 15 min at 72 °C. In the chloroplast DNA, a transcribed region of *matK* and the four intergenic spacer regions of *rps14-psaB, rpl16-rpl14, psbC-trnS, and rpl20-rps18* were amplified. The *matK* region was amplified with three primer pairs of matKAF and LR, matKMF and NR, and matKPF and 8R (Ooi et al. 1995). For the other four regions, primer pairs

of rps14 and psaB (Fofana et al. 1997), rpl16 and rpl14 (Nakamura et al. 1997), psbC and trnS (Demesure et al. 1995), and rpl20 and rps18 (Wang et al. 1999), were used respectively. The PCR cycle consisted of 1 min at 95 °C, followed by 35 cycles of 1 min at 94 °C, 1 min at 50 °C, 2 min at 72 °C, and ended with a final extension of 15 min at 72 °C for the *matK, rpl20-rps18*, and *rps14-psaB4* regions. For the *psbC-trnS* and *rpl16-rpl14* regions, the annealing temperature was changed to 48 °C. The PCR products were purified using a Wizard PCR Preps DNA purification system (Promega, Madison, USA). The purified DNA was sequenced by standard methods with a Big dye deoxy-terminator cycle sequencing kit (Perkin Elmer, Foster City, CA, USA) using a model 377 automated sequencer (Applied Biosystems, Foster City, CA, USA). The sequences were read using both the forward and reverse primers.

Phylogenetic analysis

The sequence boundaries between the two ITS regions and three ribosomal regions (18S, 5.8S, and 26S) were determined according to Baldwin (1992). The regions of 18S, 5.8S, and 26S were excluded from the analyses. ITS data of two Brazilian species (*S. ophryophylla* and *S. tenuis*), and chloroplast data of 37 Mexican species and *S. lundiana* could not be gained because of the sample shortage.

Multiple sequence alignment and phylogenetic analyses

The nucleotide sequences of nuclear ITS1, ITS2, chloroplast *matK* and four chloroplast intergenic spacers were aligned separately by E-INS-i algorithm implemented in MAFFT v7.245 (Katoh and Standley 2013). Gaps were treated as missing data. We performed maximum parsimony (hereafter MP), maximum likelihood (hereafter ML) and Bayesian phylogenetic analyses for concatenated sequences of ITS1 and ITS2, and concatenated sequences of chloroplast *matK* and intergenic spacers independently because the congruence between nuclear and chloroplast sequences was rejected (see below). ML phylogenetic trees were reconstructed with the aid of RAxML v8.2.4 (Stamatakis 2014). 100 trees were generated by random sequence addition method under MP criterion and used as starting trees in ML tree search. Bootstrap analyses (1,000 pseudoreplicates) were conducted to evaluate clade credibility under ML and MP criteria. PAUP* 4.0b10 (Swofford 2003) was used for MP bootstrap analyses. In each pseudoreplicate, one random sequence addition tree under MP criterion was used as starting tree under both criteria. The tree-bisection-reconnection branch swapping and no multiple trees saving option were used in MP bootstrap analyses. Bayesian tree inferences were conducted using MrBayes v3.2.6 (Ronquist et al. 2012). Each Metropolis-coupled Markov chain Monte Carlo analysis performed 10,000,000 generations. The sampled trees and models from first half generations were discarded as burn-in and a majority-rule consensus tree was constructed from the sampled trees from latter half generations. Convergence of likelihood values between parallel two runs were visually checked using Tracer v1.6 (Rambaut et al. 2014). Effective sample sizes of all parameters were also calculated by Tracer v1.6 (Rambaut et al. 2014). The substitution models and partitioning schemes applied in the ML and Bayesian analyses were selected by Kakusan4 v4.0.2015.01.23 (Tanabe 2011) based on Bayesian information criterion (Schwarz 1978). The alignment length was used as sample size for calculating Bayesian information criterion.

Testing phylogenetic congruence between nuclear and chloroplast sequences

The phylogenetic congruence between nuclear and chloroplast sequences was tested using incongruence length difference test (Farris et al. 1994) implemented in PAUP* 4.0b10 (Swofford 2003) and approximately unbiased (hereafter AU) test (Shimodaira 2002) implemented in CONSEL v0.20 (Shimodaira and Hasegawa 2001) which compared the log-likelihoods between independent trees hypothesis and common tree hypothesis on the subset sequence data that contains 40 operational taxonomic units shared between the full sets of nuclear and chloroplast sequence data.

Topology test

Topology-constrained ML tree searches were performed on chloroplast sequence data and nuclear sequence data using RAxML v8.2.4 (Stamatakis 2014). The log-likelihoods of the unconstrained ML tree and the constrained ML trees were compared using AU test (Shimodaira 2002) implemented in CONSEL v0.20 (Shimodaira and Hasegawa 2001). For chloroplast sequence data, each of the following three monophyletic groups were enforced: (1) the 4 annuals (*S. aschenborniana, S. ecatepecana, S. micrantha, S. mitopoda*), (2) all *Stevia* except for the 4 annuals and *S. tephrophylla*, and (3) Mexican shrub species excluding *S. tephrophylla* (designated as Mex-S below).

On the nuclear sequence data, the unconstrained ML tree supported monophyly of diploid–polyploid or polyploid–polyploid of *S. alatipes, S. connata, S. karwinskyana, S. origanoides, S. pilosa* and *S. stolonifera*, and non-monophyly of those of *S. caracasana, S. eupatoria, S. monardifolia* and *S. nepetifolia*. Therefore, ten converse topological hypotheses-enforced constrained ML tree searches were performed and the log-likelihoods were compared. Because

RAxML does not support negative topological constraint, the most frequently observed non-monophyly hypotheses in bootstrap analysis were enforced as positive topological constraints instead of negative topological constraints of monophyly.

Divergence time estimation on nuclear sequence data

Time calibrations of ML and Bayesian trees were conducted with the aid of mcmctree included in PAML v4.8a (Yang 2007). The outgroup taxa (*Ageratina* and *Mikania*) were eliminated from the data set used in phylogenetic reconstruction because the large branch lengths on the tree of those taxa might violate divergence time estimation. The 95% highest posterior density interval of estimated divergence time between *Hofmeisteria* and *Stevia*, 7.8 (3.2–13.8) Mya (see Supplementary), was used as age constraint of uniform distribution. Both upper and lower bounds were given as soft bounds (2.5% of samples beyond the bounds were allowed for each of upper and lower bounds). Independent lognormal rates model (Rannala and Yang 2007) and approximate likelihood calculation was applied. The other options and values were determined with reference to a step-by-step tutorial (Inoue et al. 2011).

Results

Sequence characteristics

Properties of the sequence data sets of each region are shown in Table 2. All of the sequenced samples had few or no polymorphisms.

Phylogenetic analysis of nuclear sequence data

A ML tree was constructed on nuclear ITS sequences from 81 *Stevia* species, *Carphochaete, Revealia, Hofmeisteria, Mikania*, and *Ageratina* (Fig. 2). Monophyly of *Stevia* was supported by all of MP, ML and Bayesian tree reconstruction (MP bootstrap support (BS) = 93%, ML-BS = 85% and Bayesian posterior probability (BPP) = 1.00). *Stevia tephrophylla* S. F. Blake was separated at the base of the genus. Four annuals, *S. aschenborniana, S. ecatepecana, S. micrantha*, and, *S. mitopoda* formed a monophyletic clade Mex-A (MP-BS = 100%, ML-BS = 100% and BPP = 1.00), which is sister to the other *Stevia* species excluding *S. tephrophylla*. Mex-S (Mexican shrub species excluding *S. tephrophylla*), also formed a monophyletic clade (MP-BS = 76%, ML-BS = 90% and BPP = 1.00). Sister clade to Mex-S was also strongly supported (MP-BS = 98%, ML-BS = 97% and BPP = 1.00) and designated as Mex-PA, which contains perennials and annuals. Four annuals (*S. ephemera, S. lita, S. mexicana*, and *S. occidentalis*) were scattered within Mex-PA. Mex-PA was subdivided into three weakly supported subclades, Mex-PA1 (MP-BS = 72%, ML-BS = 65% and BPP = 0.98), Mex-PA2 (MP-BS = 32%, ML-BS = 16% and BPP = 0.64) and Brazil, which contains all of the Brazilian species (MP-BS = 76%, ML-BS = 89% and BPP = 0.81). Brazil + *S. ephemera* and Mex-PA2 were monophyletic but the support for this clade was weak (MP-BS = 55%, ML-BS = 27% and BPP = 0.89).

Phylogenetic analysis of chloroplast sequence data

The concatenated sequences of five regions from chloroplast genomes of 33 Mexican *Stevia*, 12 Brazilian *Stevia* and three outgroup species were compiled into a ML phylogenetic tree (Fig. 3). Although the chloroplast tree was less resolved than the ITS tree, there were several aspects of clear similarity. *Stevia tephrophylla* was placed in the base of *Stevia* (MP-BS = 100%, ML-BS = 100% and BPP = 1.00), and all the other *Stevia* were monophyletic (MP-BS = 100%, ML-BS = 100% and BPP = 1.00). Monophyly of nine out of twelve Brazilian species was also supported (MP-BS = 68%, ML-BS = 68% and BPP = 0.98).

Phylogenetic congruence between nuclear and chloroplast sequences

In the incongruence length difference test, congruence between nuclear and chloroplast sequence data was rejected

Table 2 Summary of alignment properties

DNA region	Length	Aligned length	Total no. site changes	No. informative site changes	No. informative indels
ITS	717–726	751	280	167 (22.2%)	25
matK	1125	1125	61	28 (2.5%)	0
rps14-psaB	446–469	469	7	5 (1.1%)	1
rpl16-rpl14	642–681	721	15	8 (1.1%)	8
psbC-trnS	258–284	301	16	12 (4.0%)	4
rpl20-rps18	603–613	613	27	7 (1.1%)	2

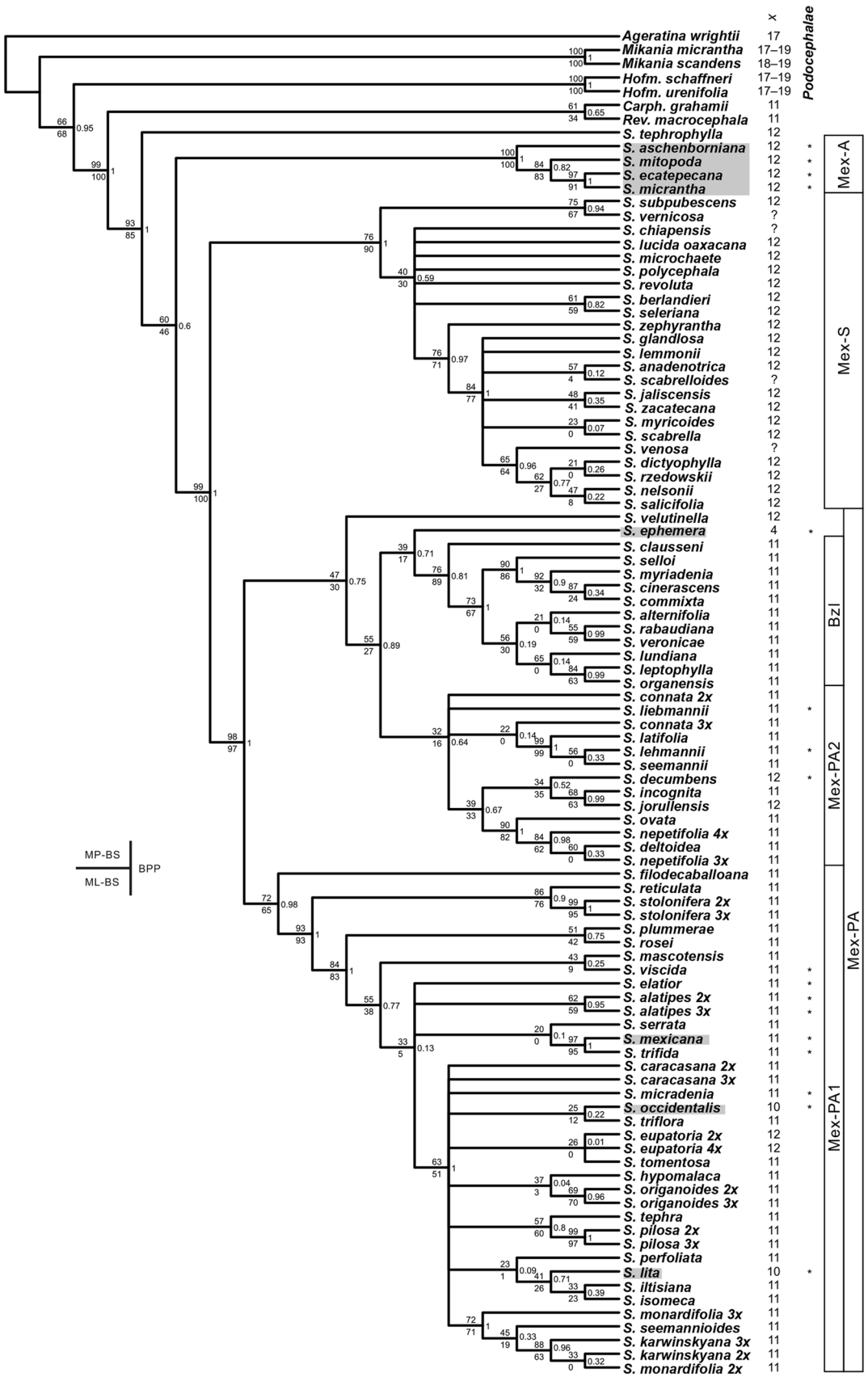

Fig. 2 ML tree based on ITS sequences. *Asterisks* (*) indicate that species belong to *Podocephalae. Gray shading* indicate annual species

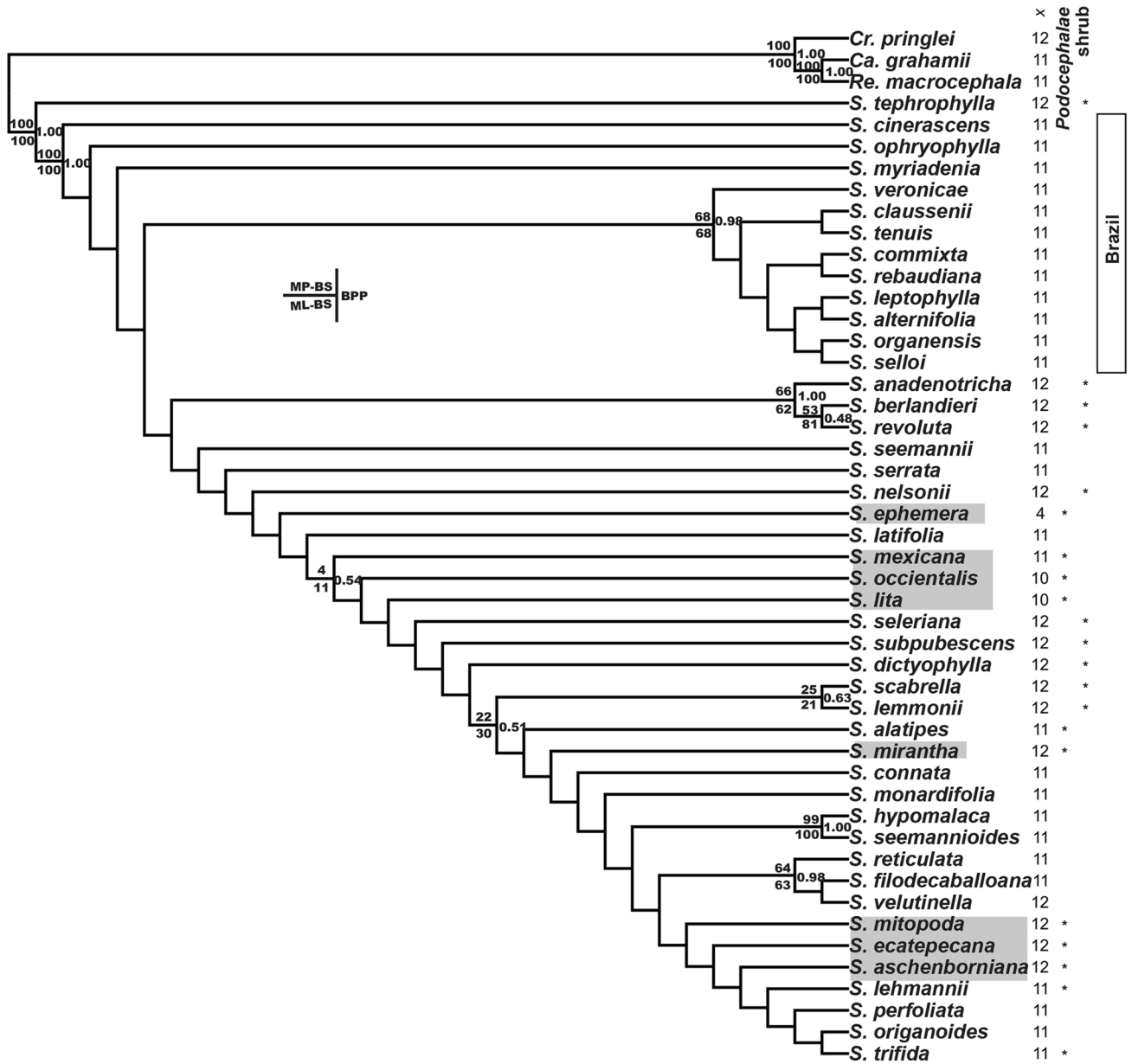

Fig. 3 ML tree based on chloroplast DNA sequences. *Asterisks* (*) indicate the species belongs to section *Podocephalae* and/or shrub species. *Gray shading* indicate annual species

($P = 0.001$). The phylogenetically congruent common tree hypothesis was also rejected by AU test ($P < 0.0001$).

Topology test

For chloroplast sequence data, the three groups, (1) the 4 annuals (*S. aschenborniana, S. ecatepecana, S. micrantha, S. mitopoda*), (2) all *Stevia* except for the 4 annuals and *S. tephrophylla*, and (3) Mex-S, monophyly was not rejected by AU test ($P = 0.590$, 0.059 and 0.456, respectively).

For the nuclear sequence data, each of the infraspecific diploid–polyploid or polyploid–polyploid pairs of *S. alatipes, S. caracasana, S. connata, S. eupatoria, S. karwinskyana, S. monardifolia, S. nepetifolia*, and *S. origanoides*, both hypotheses of monophyly and non-monophyly were not rejected by AU test ($P = 0.713$, 0.768, 0.763, 0.666, 0.544, 0.567, 0.767, 0.719 and 0.603, respectively), but for the diploid–polyploid pairs of *S. pilosa* and *S. stolonifera*, non-monophyly was rejected ($P = 0.010$ and <0.001, respectively).

Divergence times estimated based on nuclear sequence data

The time-calibrated chronogram estimated within *Stevia* using ITS ML tree topology is shown as Fig. 4. The origin of the genus was determined as 7.3 Mya [95% highest posterior density (HPD) interval: 3.0–13.4] followed by the separation of the most basally branching species, *S. tephrophylla*, at 7.0 Mya (95% HPD interval: 2.9–13.0). The shrub (Mex-S) and herb (Mex-PA) clades separated at 6.6 Mya (95% HPD interval: 2.7–12.3) (Fig. 4). The divergence time of the Brazilian clade from the Mexican species was estimated to be 5.2 Mya (95% HPD interval: 2.0–10.0).

Discussion

Incongruence between the ITS and the cpDNA tree

Whereas both the ITS and the cpDNA trees showed that *S. tephrophylla* is sister to all the remaining species of *Stevia*, there were the following major topological incongruities between the two (Figs. 2, 3): (1) the four annual species were clustered as an early branched clade (Mex-A) in the ITS tree but dispersed in a large terminal clade in the cpDNA tree, (2) all shrubs except *S. tephrophylla* formed a clade (Mex-S) in the ITS tree, but did not form a single clade in the cpDNA tree; (3) Brazilian species were placed as an inner clade nested in a larger Mexican clade

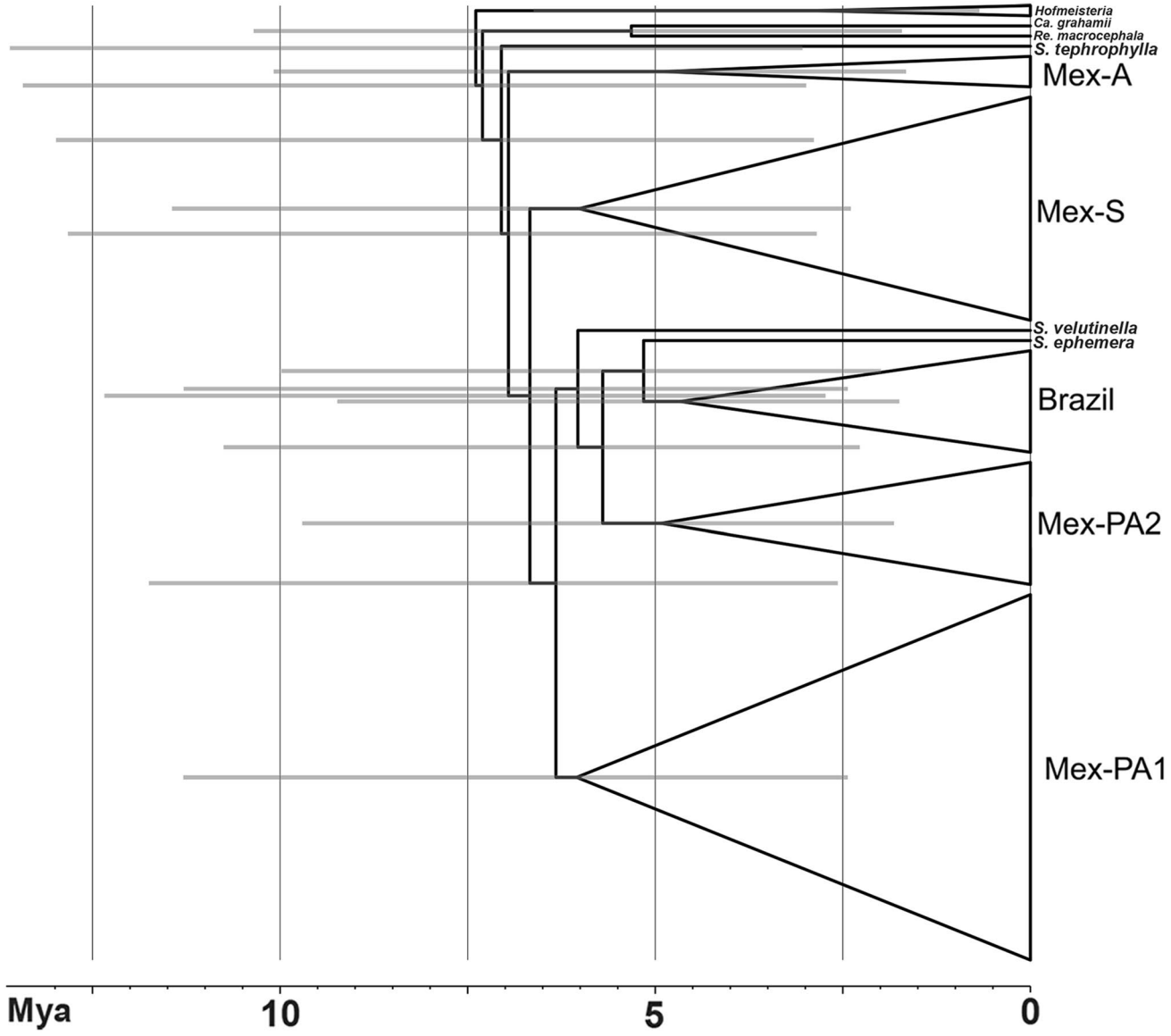

Fig. 4 Schematic representation of the ITS tree showing the estimated divergence times. The *error bars* indicate the 95% highest posterior density (HPD) intervals for the divergence times estimates

in the ITS tree, but branched near the base of the cpDNA tree. Because the phylogenetic congruence of two trees was rejected by incongruence difference test ($P=0.001$) and AU test ($P<0.0001$), phylogenetic analysis using a combined data set of ITS and cpDNA was not performed. Therefore, the following analyses were performed on the ITS tree. Compared to the ITS tree, fewer branches were strongly supported in the cpDNA tree, indicating the paucity of information of the chloroplast sequence data.

Phylogenetic relationships within *Stevia* in Mexico

The ML tree based on the ITS sequences strongly supported the monophyly of *Stevia* (Fig. 2), agreeing with the morphology-based view that *Stevia* is one of the most distinctive genera in the tribe Eupatorieae (King and Robinson 1987).

All of the shrub species, except for *S. tephrophylla*, were included in a well supported clade, Mex-S (Fig. 2). All of those shrub species are morphologically distinct, but have the same chromosome number, $2n=24$ (Grashoff 1972; Turner 1997; Watanabe et al. 2001, 2007; Watanabe 2008), and their differentiation in the ITS sequence was so small that the relationships among these species were not resolved well. Despite their morphological distinctiveness, it is likely that species diversification in these shrub species occurred rather recently. While *S. lucida* Lagasca, *S. salicifolia* Cav., and *S. subpubescens* Lagasca are widely distributed, other shrub species have very narrow and isolated distribution ranges, suggesting that those species diverged under geographical isolation.

The Mex-PA clade, a sister clade to Mex-S, comprises herb species, including both perennials and annuals. This clade includes many perennial species with the basic chromosome number of $x=11$ and a few perennial species with $x=12$ (Fig. 2). The Mex-PA clade was divided into two major subclades, Mex-PA1 and another clade comprising Mex-PA2, *S. ephemera, S. velutinella*, and Brazilian species. Within both of these two subclades, the relationships were not well resolved, suggesting recent speciation in these subclades.

The eight annual Mexican species exhibited variation in their chromosome number from $x=4$–12 (Watanabe et al. 2001, 2007; Watanabe 2008). Four species, *S. aschenborniana, S. ecatepecana, S. micrantha*, and *S. mitopoda*, in which $x=12$, formed a clade (Mex-A) branching near the base of the tree (Fig. 2). Four other annual species, *S. ephemera* ($n=4$), *S. lita* ($n=10$), *S. mexicana* ($n=11$), and *S. occidentalis* ($n=10$), were scattered in the larger herb clade (Mex-PA) (Fig. 2). *Stevia occidentalis* (Fig. 1c) is morphologically distinguishable from *S. aschenborniana* (Fig. 1b) only by having glandular hairs (not glandular in *S. aschenborniana*) and had been treated as a variety of the latter (Grashoff 1972). However, recent studies showed that those have different chromosome numbers: $2n=20$ in *S. occidentalis* vs. $2n=24$ in *S. aschenborniana* (Soejima et al. 2001b; Watanabe et al. 2001). In the ITS tree, *S. occidentalis* was included in a derivative subclade of Mex-PA1 with another annual, *S. lita* (MP-BS = 63%, ML-BS = 51%, and BPP = 1.00), supporting the close relationship of *S. occidentalis* and *S. lita* both of which have glandular hairs on stems and leaves and the same chromosome number ($2n=20$). Another annual species, *S. mexicana* was sister to a perennial species *S. trifida* (Fig. 2) both of which have the same chromosome number ($2n=22$) and deeply dissected leaves (Fig. 1d), a trait that is not observed in any other Mexican *Stevia* species. Based on these results, we suggest that *S. mexicana* originated from a *S. trifida*-like perennial ancestor and not from the other annuals.

It is difficult to determine the ancestral chromosome number in *Stevia* because the chromosome number of the closely related genera, *Carphochaete* and *Revealia*, is $n=11$, whereas it is $n=12$ in the most basally branching species of *Stevia* (*S. tephrophylla*). However, all of the species of Mex-A and Mex-S, which are divided near the base, also have $n=12$, so it is likely that the ancestral chromosome number of *Stevia* is $n=12$. In the tribe Eupatorieae, lower chromosome numbers are derived from higher chromosome numbers (Ito et al. 2000; Semple and Watanabe 2009; Watanabe et al. 1995). The chromosome number in *Stevia* also appears to have evolved from high ($n=12$) to low ($n=4$).

In *Stevia*, some infrageneric groups have been recognized previously based on the shape of their inflorescences. Robinson (1930) divided the North American *Stevia* into two sections: *Podocephalae* Schultz-Bip., which possesses lax disposed long-pedicellate heads, and *Corymbosae* Schultz-Bip., which possesses heads in dense corymbose clusters. *Corymbosae* have been further divided into two subseries: *Fruticosae* comprising shrub species and *Herbaceae* comprising perennials and annuals (Robinson 1930). Later, Grashoff (1972) recognized three groups at the sectional level (*Podocephalae, Corymbosae*, and *Fruticosae*). All of the shrub species examined in the present study, except for *S. tephrophylla*, constituted a single clade, Mex-S (Fig. 2). Therefore, the Mex-S clade supports the phylogenetic cohesion of *Fruticosae*, while the species from sections *Podocephalae* and *Corymbosae* were intermingled in Mex-PA (Fig. 2), and thus monophyly of two groups were not supported.

Estimation of the origin and the divergence times of Stevia

The divergence time between the *Stevia* clade and *Hofmeisteria* was estimated to be 7.8 (3.2–13.8) Mya

(see Supplementary), and the divergence time between *Carphochaete* + *Revealia* and *Stevia* were estimated as 7.3 (3.0–13.4) Mya (Fig. 4). The most basally branching species, *S. tephrophylla*, branched at 7.0 (2.9–13.0) Mya, so the origin of *Stevia* might have been between 7.0 and 7.3 Mya. Because S. *tephrophylla* is endemic to Mexico and the closely related genera *Carphochaete* and *Revealia* are also mostly restricted to Mexico (King and Robinson 1987), it is likely that *Stevia* originated in Mexico. The habit of *S. tephrophylla* is very similar to that of the species of *Carphochaete* and *Revealia*, in that all of them are small shrubs lacking a main stem and having dormant buds only near the ground. In addition, both *Carphochaete* and *S. tephrophylla* are found in open understory of dry mountain forests dominated by pines and oaks. These states might represent the ancestral habit and habitat of *Stevia*.

In the age that *Stevia* originated, the climate changed toward increasing aridity and decreasing temperature (Axelrod 1958; Graham 1989; Valiente-Banuet et al. 1998). In Mexico, the Sierra Madre Occidental and the Neovolcanic belt (Trans-Mexican Volcanic Belt) were developing during the Oligocene and early Miocene, and the last uplift of the Sierra Madre Occidental occurred between 24 and 15 Mya (Ferrari et al. 1999, 2000; see; Becerra 2005). Although Graham (1999) noted that the northern temperate vegetation, which comprised pines and oaks, was sparsely distributed in Mexico during Miocene, a favorable habitat for *Stevia* must have been established before the origin of the genus.

Grashoff (1972) proposed a southern hemisphere origin for *Stevia*, but our results indicated a northern origin and a subsequent southward migration. Eleven Brazilian species formed a clade that was nested within the Mexican herbs. The time of the node that separated the Brazilian clade from the Mexican herbs was estimated to be 5.2 (2.0–10.0) Mya (Fig. 4). The age of the formation of the Isthmus of Panama has long been considered about 3–3.5 Mya (Gentry 1982; Keigwyn 1978; Leigh et al. 2014), but recently, a geochronological study of Montes et al. (2015) estimated the closure of the Central American Seaway happened in middle Miocene, and Bacon et al. (2015) indicated it has occurred between ca. 23–20 and 8–6 Mya using molecular data combined with fossil records. Because the pappus of *Stevia* is constructed with bristle-like awns, a crown of small scales, or is absent, the achene of *Stevia* is not suitable for wind dispersal. The land connection provided *Stevia* a good opportunity for intercontinental dispersal. Our samples from South America are limited and it remains uncertain whether all the species of *Stevia* endemic to South America are monophyletic. Further analyses using more samples from South America, especially from other regions as Argentina and Paraguay, each has 33 and 20 species of *Stevia*, respectively, are necessary to better understand the migration history of *Stevia*.

A few *Stevia* species are found in both North and South America. At least eight species (*S. caracasana, S. elatior, S. incognita, S. lehmannii, S. lucida, S. nepetifolia, S. serrata*, and *S. triflora*) are distributed predominantly in Mexico, but also range throughout Central America into South America. With the exceptions of *S. lehmannii* (diploid perennial, $n = 11$) and *S. lucida* (diploid shrub, $n = 12$), these species are all polyploid agamosperms, which are found in pastures or along roadsides. Thus, the expansion of these agamospermous species may have been associated with human disturbance. A paleoecological analysis conducted in lowland Panama (Bush et al. 1992) supports this possibility based on evidence of a rapid change in vegetation, which was caused by the beginning of human activity approximately 11,000 years ago.

Adaptive radiation in Mexican *Stevia*

In Mexican *Stevia*, as well as the other genera of Asteraceae in Mexico such as *Baccharis, Brickellia, Dahlia*, and *Senecio*, the majority of species diversity is found in pine–oak forests (Rzedowski and Rzedowski 2001). Schilling et al. (2015) estimated the origin of *Brickellia* as 9 Mya, and showed that it actively diversified during times of significant changes in climatic conditions and orography from late Miocene to present. Our estimate for the divergence time between *Stevia* and *Hofmeisteria* as 7.8 (3.2–13.8) Mya is close to the origin of *Brickellia*. According to Turner (1997), more than 70% of the shrub species and over 75% of the herb species of *Stevia* are found in pine–oak forests, and approximately 10% of the shrub and 8% of the herb species are found in conifer or cloud forests located at higher altitudes. A few species grow at lower altitudes, such as *S. jaliscensis*, a shrub found in tropical deciduous forests, as well as *S. lasioclada* (perennial) and *S. mitopoda* (annual), both of which are found in mixed subtropical forests (Turner 1997). It seems that a habitat shift from the pine–oak zone to other vegetation zones has not happened frequently, but *Stevia* occupies various habitats such as forest understories, grasslands, dry slopes, dry lava, meadows, ravines, and glades. This habitat variation would have contributed to the species diversity of Mexican *Stevia*.

In contrast to the wide distribution of pine–oak forest in Mexico, many *Stevia* species have narrowly restricted distributions. The following species are found only at their type localities or at a few localities distributed within small areas of Mexico and Guatemala: *S. anadenotricha, S. chiapensis, S. scabrella, S. seleriana, S. revoluta, S. rzedowskii, S. venosa, S. vernicosa*, and *S. zephyrantha* among the shrubs; and *S. decumbens, S. karwinskyana, S. liebmannii,*

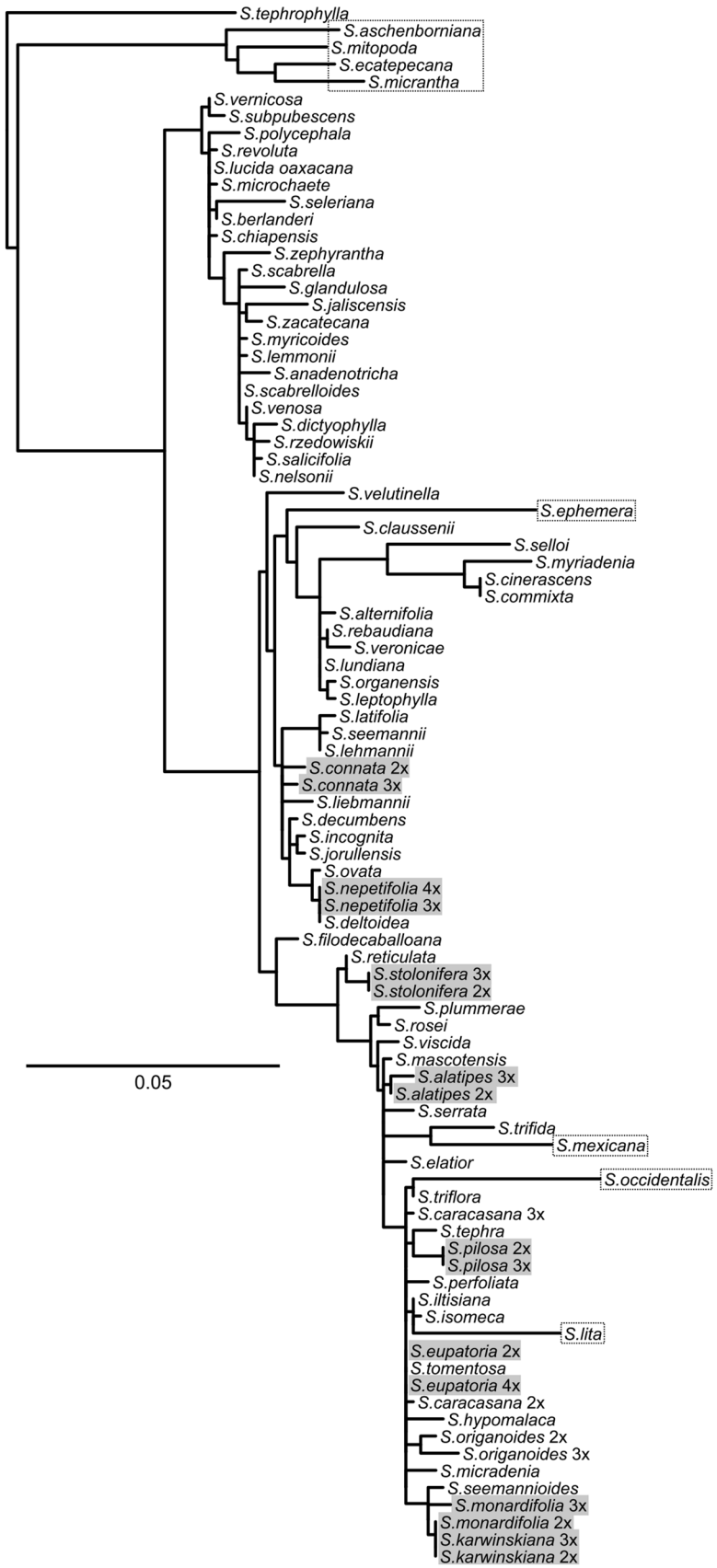

Fig. 5 Majority-rule consensus tree obtained by Bayesian analyses based on the ITS sequences. The *shadow squares* indicate conspecific pairs

S. lita, S. mitopoda, S. perfoliata, S. reticulata, S. rosei, S. seemannii, S. seemannioides, and *S. stolonifera* among the herbs. Pine–oak forest has existed in Mexico since the Miocene and expanded greatly into Panama during the Pliocene (Graham 1999). During this habitat expansion, *Stevia* might have extended its distribution area and species diversity increased. However, climate fluctuations during the late Pleistocene and Holocene caused frequent vegetation changes in Mexico (Lozano-García et al. 2005; Mastretta-Yanes et al. 2015). Under those changes, the habitats of *Stevia* in Mexico would have been repeatedly fragmented and thereby many species would have been restricted to small distribution areas.

Origin of agamospermous polyploids

Among 10 conspecific pairs of different ploidy levels, six pairs formed conspecific clades: *S. alatipes, S. karwinskyana, S. nepetifolia, S. origanoides, S. pilosa*, and *S. stolonifera*, while four other diploid–polyploid pairs (*S. caracasana, S. connnata, S. eupatoria*, and *S. monardifolia*) were paraphyletic with other species (Fig. 5). The results of AU strongly indicated the monophyly of diploid and triploid in *S. pilosa* and *S. stolonifer*. For other eight conspecific pairs, both monophyly and non-monophyly was not rejected. The results might reflect the limited number of informative sites that can be found among closely related species. Hybridization between closely related species could also explain these examples of polychotomy, but the diploids and polyploids of these pairs were morphologically similar and no sequences were found with peaks of mixed bases indicating heterozygotes. These polyploids studied here are probably autopolyploids, suggesting that interspecific hybridization did not play a major role in the evolution of agamospermous polyploids in *Stevia*. However, it has been suggested that some agamospermous species of *Stevia* may include allopolyploids with morphological evidence of hybridization with related species (Grashoff 1972; Soejima et al. 2001a; Watanabe et al. 2001, 2007). Thus, further research is needed to demonstrate whether the allopolyploid origin of agamosprems is also the case in *Stevia* as in many other plant groups comprising agamospermous polyploids (Stebbins 1950; Doyle et al. 2008).

Conclusion and future scope

The ITS sequences of 70 Mexican and 13 Brazilian species enabled us to reconstruct the time-calibrated chronogram for the history of divergence in the genus *Stevia*. There are three major findings from this chronogram. First, the origin of *Stevia* around 7.0–7.3 million years ago (Mya) corresponded to the end of Miocene, when the climate changed toward increasing aridity and decreasing temperature. Second, a clade of shrubs and a clade of herbs diverged at about 6.6 Mya. Third, a Brazilian clade was originated at about 5.2 Mya indicating the migration from North America to South America. However, we could not determine more recent divergence times due to limited resolution of the ITS tree among closely related species. The poor resolution for many *Stevia* species both in shrub and herb clades suggests that they diverged recently. Further molecular analyses using more markers with higher evolutionary rates are needed to reconstruct the time-calibrated chronogram for these closely related species. Many of these species have restricted distributions and narrow habitat preferences. Thus, detailed molecular analyses will contribute to deepen our understanding of the mechanism of adaptive divergence in general.

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